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RENEWABLE BIO-SOLAR HYDROGEN PRODUCTION: THE SECOND GENERATION (Part B)

Don Bryant
PENNSYLVANIA STATE UNIVERSITY

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Final Report

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14. ABSTRACT

In this project we used targeted mutagenesis, overexpression of genes, transcription profiling, and metabolic profiling of the cyanobacterium Synechococcus sp. PCC 7002 to understand better how to optimize the production of biohydrogen and potential carbon-based biofuel molecules. A major finding was that the tricarboxylic acid (TCA) cycle is not branched, as had been believed for nearly 50 years, but is in fact closed by two non-canonical enzymes that replace 2-oxoglutarate dehydrogenase. In addition to redefining the TCA cycle, we validated the occurrence of the glyoxylate cycle and the gamma-aminobutyric acid (GABA) shunt in a few cyanobacteria, and we studied the partitioning of metabolites between the glycolytic and oxidative pentose phosphate pathways. Important transcription regulators, including RbcR, Fur, and ChlR, were identified and characterized. Approaches to maximize biohydrogen production and carbon-based biofuels (e.g., cellulose, poly-hydroxybutyrate) were characterized and optimized. These studies yielded important new biochemical details about critically important aspects of cyanobacterial physiology and metabolism.

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1. **PI: Donald A. Bryant**
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5. **Institution: The Pennsylvania State University**
6. **Inventions/Patents: None**
7. **Scientific Personnel Supported**
    1. Dr. Donald A. Bryant, Principal Investigator
    2. Dr. Marcus Ludwig (Postdoctoral associate)
    3. Dr. Zhongkui Li (Postdoctoral associate)
    4. Dr. Chi (George) Zhao (Postdoctoral associate)
    5. Dr. Gaozhong Shen (Senior Research Associate)
    6. Mr. Shuyi Zhang (Ph. D. student)
    7. Mr. Ming-Yang Ho (Ph. D. student)
    8. Ms. Chyue Yie Chew (Honors Undergraduate Researcher)
    9. Mr. Yang Liu (Honors Undergraduate Researcher and Research Assistant)
    10. Ms. Tiing Tiing Chua (Undergraduate Researcher)

8. **Summary of Key Finding/Results/Accomplishments:**

Abstract of report

Synechococcus sp. PCC 7002 is a euryhaline, unicellular cyanobacterium that has many desirable properties for a platform for metabolic engineering for solar biofuel production. It is one of the fastest growing cyanobacteria known, tolerates high irradiance levels and oxidative stress very well, has a completely sequenced genome, and is readily amenable to genetic manipulations. In this project we used targeted mutagenesis, overexpression of homologous and heterologous genes, transcription profiling, and metabolic profiling to understand better how to optimize the production of biohydrogen and potential carbon-based biofuel molecules in this cyanobacterium. A major discovery was that the tricarboxylic acid (TCA) cycle is not branched, as had been believed for nearly 50 years, but is in fact closed by two non-canonical enzymes that replace 2-oxoglutarate dehydrogenase. In addition to redefining the TCA cycle, we validated the occurrence of the glyoxylate cycle and the gamma-aminobutyric acid (GABA) shunt in a few cyanobacteria, and we studied the partitioning of metabolites between the glycolytic and oxidative pentose phosphate pathways. Important transcription regulators, including RbcR, Fur, and ChlR, were identified and characterized, and a global model of the transcription network was generated. Approaches to maximize biohydrogen production and carbon-based biofuels (e.g., cellulose, poly-hydroxybutyrate) were characterized and optimized. These studies yielded important new biochemical details about critically important aspects of cyanobacterial physiology and metabolism. This project supported the training of three postdoctoral scientists, two Ph. D. students, two undergraduate honors students, and one other undergraduate researcher. To date, seventeen papers (including one in *Science*) have been published or submitted, and seven papers based on completed research are currently being prepared for publication.

The numbers shown in bold font in brackets **[XX]** refer to the numbers of the publications in the list that follows.

A. TCA cycle in cyanobacteria

Undoubtedly, a high point of our AFOSR-supported research has been our work on the TCA cycle in Synechococcus sp. PCC 7002 and other cyanobacteria. In a study published in *Science* **[5]**, we disproved the long-held notion that the TCA cycle in cyanobacteria is incomplete because of the absence of 2-oxoglutarate dehydrogenase. While it is true that this enzyme is missing in all characterized cyanobacteria **[13]**, most cyanobacteria have two other enzymes, 2-oxoglutarate decarboxylase and succinic semialdehyde dehydrogenase, that complete the TCA cycle. In follow-on work to our initial studies, we verified that *Chlorogloeopsis fritschii* has isocitrate lyase and malate synthase, and thus has a complete glyoxylate cycle for enhanced acetate utilization **[16]**. Only few other cyanobacteria have these enzymes, however, and thus acetate utilization by this pathway is not common in cyanobacteria. We demonstrated that this capability could be transferred to *Synechococcus* sp. PCC 7002 by heterologous expression of the required genes, and showed that this could enhance the capacity for this strain for poly-beta-hydroxybutyrate production **[20]**. In *C. fritschii*, the glyoxylate cycle also appears to be associated with poly-beta-hydroxybutyrate synthesis and carbon storage. Finally, we also validated the long-postulated activities of the GABA shunt by overproducing and assaying the enzymes for this pathway from *Synechocystis* sp. PCC 6803 **[19]**. Similar to the situation with the glyoxylate cycle, a key enzyme of the GABA shunt is only found in a few cyanobacteria. Thus, for the majority of cyanobacteria, the TCA cycle is a cycle only because of the enzymes that we identified **[5]**. We have recently conducted metabolomics studies of intermediates in the TCA cycle in the wild type and mutants of *Synechococcus* sp. PCC 7002 that lack key enzymes of the TCA cycle **[19]**. These studies showed the anticipated accumulation patterns for intermediates and for the first time demonstrated the occurrence of succinic semialdehyde as a metabolic intermediate in cells. Interestingly, marine *Synechococcus* spp. and *Prochlorococcus* spp. apparently are the only cyanobacteria with incomplete TCA cycles **[13]**.

B. Global Transcription Profiling by RNA-seq

Dr. Marcus Ludwig developed all the methods necessary to perform global transcription profiling in *Synechococcus* sp. PCC 7002. All of the initial studies were performed on the SOLiD platform, but more recently we have shifted to an Illumina HiSeq platform and implemented a highly successful ribosomal RNA depletion protocol. This reduces the cost per sample and increases the sequence depth per sample dramatically. In a series of publications **[3, 6, 7, 23]**, Dr. Ludwig explored global transcription changes in response to changes in physico-chemical environment parameters, including temperature, redox, light intensity, darkness, anoxic conditions, nutrient limitation, etc. These data provided many useful insights into how to manipulate central metabolic pathways to enhance hydrogen production. These data have recently been analyzed to produce a global transcription network model for this cyanobacterium **[17]**. At least some predictions from this model concerning the *fur* regulon have been validated in studies of a knock-down mutant in the *fur* gene **[23]**. Transcription profiling also identified a gene regulator, ChlR, which activates transcription from a single promoter under microoxic/anoxic conditions **[11]**. This regulator works in both *E. coli* and *Synechococcus* sp. PCC 7002 and could be useful for controlling the expression of oxygen-sensitive enzymes such

as hydrogenase. Global transcription profiling analysis has now been incorporated into my lab as a routine method that has been extremely helpful in characterizing other mutants and physiological processes occurring in other cyanobacteria. For example, we showed that acetate has only a very minor effect on the transcript levels (2- to 3-fold increase) of the *aceAB* genes of the glyoxylate cycle **[16]**. Another example came from transcription profiling of an *rbcR* null mutant strain. This mutant surprisingly showed enhanced expression of the bidirectional hydrogenase, and correspondingly, the *rbcR* mutant strain showed enhanced hydrogen evolution under various conditions. By combining this mutation with other previously identified mutations that enhance hydrogen production, a strain with a high rate of hydrogen production could be constructed.

C. (Over)-expression of genes in *Synechococcus* sp. PCC 7002

One of the most powerful developments from our MURI-funded research was the development of tools for expression and over-expression of genes in *Synechococcus* sp. PCC 7002 **[1]**. This technology has been used repeatedly in demonstrating complementation of mutant strains, in various heterologous gene expression studies, and in overproduction of various cyanobacterial and foreign proteins. A good example of this is the hyper-production of bacterial type-1 cellulose **[14]**. We showed that cellulose is a normal component of the cell wall in *Synechococcus* sp. PCC 7002, and in a null mutant lacking cellulose synthase (CesA), up to 15% of the dry weight of cells can be cellulose when six genes from *Gluconacetobacter xylinus* are heterologously expressed at high levels in cells grown at low ionic strength **[14]**. This cellulose could be used for a variety of purposes, including production of ethanol or other alcohols or as a biomaterial. Another example is exemplified by studies on fatty acid reductase and fatty aldehyde decarbonylase **[24]**. The reductase enzyme produced in *Synechococcus* sp. PCC 7002 is much more active than that produced in *E. coli*, and the yields are sufficiently high that crystallization trials are in progress. Preliminary results have identified conditions for crystallization of these proteins from a thermophilic cyanobacterium. On the other hand, yields of the decarbonylase apoprotein are very high, but the yield of holoprotein is lower than for *E. coli*. We are continuing to study this problem in hopes of optimizing the yields of both (or finding a combination of enzymes that both exhibit high activities). This work suggests that the machinery for inserting iron into the enzyme is not performing as well as it should to maximize the yield of holoprotein.

D. Metabolic engineering to enhance the production of hydrogen or reductants

We studied a variety of cyanobacteria with diverse physiological properties and mutant strains of *Synechococcus* sp. PCC 7002 constructed with the goal of testing numerous hypotheses to maximize hydrogen production. Many of these studies were conducted with collaborators from the MURI team, especially the Dismukes laboratory. These studies helped to define those conditions that would maximize hydrogen production (e.g., see **[2, 4, 8, 9, 10, 12, 15, 18, 21 and 22]**. Because the reduction of protons and CO_2 both require a source of electrons, conditions that maximize one product can nevertheless provide useful information about the other process as well. This was an obvious outcome of studying mutant unable to store reduced carbon as glycogen, which showed a compensatory increase in the amount of compatible solutes that are produced. This observation allowed us to formulate a simple model of reductant distribution to major products in the cell **[8, 9, 15]**.

9. Archival Publications (Published) during the reporting period

1. Xu, Y., Alvey, R. M., Byrne, P. O., Graham, J. E., Shen, G. and **Bryant**, D. A. 2011. Expression of genes in cyanobacteria: adaptation of endogenous plasmids as platforms for high-level gene expression in *Synechococcus* sp. PCC 7002. *Methods Mol. Biol.* **684**: 273-293.
2. McNeely, K., Xu, Y., Ananyev, G., Bennette, N., **Bryant**, D. A., and Dismukes, G. C. 2011. *Synechococcus* sp. strain PCC 7002 *nifJ* mutant lacking pyruvate:ferredoxin oxidoreductase. *Appl. Environ. Microbiol.* **77**: 2435-2444.
3. Ludwig, M. and **Bryant**, D. A. 2011. Transcription profiling of the cyanobacterium *Synechococcus* sp. PCC 7002 using high-throughput cDNA sequencing. *Front. Microbio.* **2**:41.
4. Carrieri, D., Ananyev, G., Lenz, O., **Bryant**, D. A., and Dismukes, G. C. 2011. A sodium ion gradient contributes to energy conservation during fermentation in the cyanobacterium *Arthrospira (Spirulina) maxima* CS-328. *Appl. Environ. Microbiol.*, **77**: 7185-7194.
5. Zhang, S. and **Bryant**, D. A. 2011. The cyanobacterial tricarboxylic acid cycle. *Science* **334**: 1551-1553.
6. Ludwig, M. and **Bryant**, D. A. 2012. Acclimation of the global transcriptome of the cyanobacterium *Synechococcus* sp. strain PCC 7002 to nutrient limitations and alternative nitrogen sources. *Front. Microbio.* **3**:145.
7. Ludwig, M. and **Bryant**, D. A. 2012. Acclimation of the *Synechococcus* sp. strain PCC 7002 transcriptome to temperature, salinity and mixotrophic growth conditions. *Front. Microbio.* **3**: 354.
8. Xu, Y., Guerra, L. T., Li, Z., Dismukes, G. C. and **Bryant**, D. A. 2013. Altered carbohydrate metabolism in glycogen synthase mutants of *Synechococcus* sp. strain PCC 7002. *Metab. Eng.* **16**: 56-67.
9. Guerra, L. T., Xu, Y., Bennette, N., McNeely, K., **Bryant**, D. A., Dismukes, G. C. 2013. Metabolic analysis of an ADP-glucose pyrophosphorylase deficient mutant of *Synechococcus* sp. PCC 7002: Evidence that glycogen is the preferred substrate during auto-fermentation. *J. Biotech.* **166**: 65-75.
10. Kumaraswamy, G. K., Guerra, T., Qian, X., Zhang, S., **Bryant**, D. A. and G. C. Dismukes. 2013. Reprogramming the glycolytic pathway for increased hydrogen production in cyanobacteria: metabolic engineering of NAD^+-dependent GAPDH. *Energy Environ. Sci.* **6**: 3722-3731.
11. Ludwig, M., Pandelia, M.-E., Chew, C. Y., Golbeck, J. H., Krebs, C., and **Bryant**, D. A. 2014. ChlR protein of *Synechococcus* sp. PCC 7002 is a transcription activator that uses an oxygen-sensitive [4Fe-4S] cluster to control genes involved in pigment biosynthesis. *J. Biol. Chem.* **289**: 16624-16639.
12. Therien, J. B., Zadvornyy, O., Posewitz, M. C., **Bryant**, D. A. and Peters, J. W. 2014. Growth of *Chlamydomonas reinhardtii* in acetate-free medium when co-cultured with alginate-encapsulated strains of *Synechococcus* sp. PCC 7002. *Biotech. Biofuels.* **7**: 154.
13. Zhang, S. and **Bryant**, D. A. 2014. Learning new tricks from an old cycle: the TCA cycle of cyanobacteria, algae and plants. *Perspect. Phycol.* **1**: 73-86.

14. Zhao, C., Li, Z., Li, T., Zhang, Y., **Bryant**, D. A. and Zhao, J. 2015. High-yield production of extracellular type-I cellulose by the cyanobacterium *Synechococcus* sp. PCC 7002. *Cell Discovery*, in press.
15. Jackson, S. A., Eaton-Rye, J. J., **Bryant**, D. A., Posewitz, M. C. and Davies, F. K. 2015. Absence of global nitrogen deprivation responses in the *Synechococcus* sp. PCC 7002 glycogen-deficient *ΔglgC* mutant. *The Plant Cell*, submitted for publication.
16. Zhang, S. and **Bryant**, D. A. 2015. Biochemical validation of the glyoxylate cycle in the cyanobacterium *Chlorogloeopsis fritschii* strain PCC 9212. *J. Biol. Chem.*, submitted for publication.
17. McClure, R. S., Overall, C. C., Hill, E., Markille, L. M., McCue, L. A., McDermott, J. E., Nelson, W., Taylor, R. C., Ludwig, M., **Bryant**, D. A., Konopka, A., and Beliaev, A. S. 2015. Global transcriptome and regulatory analysis of *Synechococcus* sp. PCC 7002 from a compendium of RNA-seq data. *Genome Res.*, submitted for publication.

10. Manuscripts in preparation (based on work completed during the reporting period)

18. Kumaraswamy, G. K., Krishnan, A., Ananyev, G., **Bryant**, D. A. and Dismukes, G. C. 2015. Rewiring cyanobacterial hydrogen metabolism to elevate and reroute NAD(P)H for H_2 production. Manuscript in preparation.
19. Zhang, S., Qian, X., Dismukes, C. G, and **Bryant**, D. A. 2015. Biochemical and metabolic studies of the GABA shunt in cyanobacteria. Manuscript in preparation.
20. Zhang, S. and **Bryant**, D. A. 2015. Metabolic engineering for the production of poly-hydroxybutyrate in *Synechococcus* sp. PCC 7002. Manuscript in preparation.
21. Qian, X., Kumaraswamy, G. K., Zhang, S., Gates, C., **Bryant**, D. A. and Dismukes, C. G. 2015. Inactivation of nitrate assimilation enhances hydrogen production in the cyanobacterium *Synechococcus* sp. PCC 7002. Manuscript in preparation.
22. Krishnan, A., Zhang, S., Liu, Y., **Bryant**, D. A. and Dismukes, C. G. 2015. Metabolic engineering leads to enhancement of hydrogen production in *Synechococcus* sp. PCC 7002. Manuscript in preparation.
23. Ludwig, M., Chua, T. T., Chew, C. Y., **Bryant**, D. A. 2015. Regulation patterns of Fur-type transcription repressors in the cyanobacterium *Synechococcus* sp. PCC 7002 Manuscript in preparation.
24. Liu, Y., Zhang, S., and **Bryant**, D. A. 2015. Characterization of fatty aldehyde decarbonylase and fatty acid reductase after heterologous expression in the cyanobacterium *Synechococcus* sp. PCC 7002. Manuscript in preparation.

Changes in research objectives

The original MURI project, first funded in 2005, was entirely focused on biohydrogen, but research by the MURI team showed that hydrogen was almost exclusively produced as a by-product of fermentation in cyanobacteria. Because of the oxygen sensitivity of hydrogenases, and because of the very slow metabolic rates exhibited during fermentation in cyanobacteria, we began to turn our attention to carbon-based biofuel products. However, we continued to search for ways to improve biohydrogen production and indeed identified several ways to enhance hydrogen production during the current funding period.

Change in AFOSR Program Manager, if any:

The original MURI project was funded and administered by Dr. Walter Kozumbo, and the current 3-year award was made while Dr. Kazumbo was still the program administrator. However, just before the initiation of this project, Dr. Kazumbo unexpectedly decided to retire. At that time, Dr. Patrick Bradshaw took over the administration of the project.

1.

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Donald A. Bryant

Program Manager

The AFOSR Program Manager currently assigned to the award

Patrick Bradshaw

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12/31/2014

Abstract

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Archival Publications (published) during reporting period:

Xu, Y., Alvey, R. M., Byrne, P. O., Graham, J. E., Shen, G. and Bryant, D. A. 2011. Expression of genes in cyanobacteria: adaptation of endogenous plasmids as platforms for high-level gene expression in Synechococcus sp. PCC 7002. Methods Mol. Biol. 684: 273-293.

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9. Guerra, L. T., Xu, Y., Bennette, N., McNeely, K., Bryant, D. A., Dismukes, G. C. 2013.

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11. Ludwig, M., Pandelia, M.-E., Chew, C. Y., Golbeck, J. H., Krebs, C., and Bryant, D. A. 2014. ChlR protein of Synechococcus sp. PCC 7002 is a transcription activator that uses an oxygen-sensitive [4Fe-4S] cluster to control genes involved in pigment biosynthesis. J. Biol. Chem. 289: 16624-16639.
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Changes in research objectives (if any):

The original MURI project, first funded in 2005, was entirely focused on biohydrogen, but

research by the MURI team showed that hydrogen was almost exclusively produced as a by-product of fermentation in cyanobacteria. Because of the oxygen sensitivity of hydrogenases, and because of the very slow metabolic rates exhibited during fermentation in cyanobacteria, we began to turn our attention to carbon-based biofuel products. However, we continued to search for ways to improve biohydrogen production and indeed identified several ways to enhance hydrogen production during the current funding period.

Change in AFOSR Program Manager, if any:

The original MURI project was funded in 2005 and was administered by Dr. Walter Kozumbo, and the current 3-year award was made while Dr. Kazumbo was still the program administrator. However, just before the initiation of this project, Dr. Kazumbo unexpectedly decided to retire. At that time, Dr. Patrick Bradshaw took over the administration of the project.

Extensions granted or milestones slipped, if any:

We asked for, and were granted, a 6-month no-cost extension to allow Mr. Shuyi Zhang to complete most of his Ph. D. research, which he has done. He is now working on his dissertation and the submission of his completed work. Dr. George Zhao has largely completed follow-on studies on cellulose production as well, and his paper on this topic is in press in the inaugural issue of Cell Discovery, which should be published in the next month or so.

AFOSR LRIR Number

LRIR Title

Reporting Period

Laboratory Task Manager

Program Officer

Research Objectives

Technical Summary

Funding Summary by Cost Category (by FY, $K)

	Starting FY	FY+1	FY+2
Salary			
Equipment/Facilities			
Supplies			
Total			

Report Document

Report Document - Text Analysis

Report Document - Text Analysis

Appendix Documents

2. Thank You

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